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Efficient chemoenzymatic synthesis of sialyl Tn-antigen and derivatives[†]

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Abstract

An N-terminal and C-terminal truncated recombinant α 2–6-sialyltransferase cloned from *Photobacterium* sp. JH-ISH-224, Psp2,6ST(15–501)-His₆, was shown to be an efficient catalyst for one-pot three-enzyme synthesis of sialyl Tn (STn) antigens and derivatives containing natural and non-natural sialic acid forms.

Increased sialylation of cell surface glycoconjugates in malignant tumors has been shown to correlate to the invasive and metastatic growth of carcinoma cells.¹ Many sialic acid-containing glycans are considered as tumor-associated carbohydrate antigens (TACAs)² including sialyl Tn, sialyl T, sialyl Lewis^x, and sialyl Lewis^a, etc.³ Among TACAs, sialyl Tn antigens (Sia α 2–6GalNAc α 1-*O*-Ser/Thr) are of particular interest.⁴ The level of STn expression is limited on normal cells but is elevated on a wide range of cells of breast, prostate, pancreas, colorectal, lung, gastric, and ovarian cancers.⁵ The expression of STn has also been identified as an independent indicator for poor prognosis of cancer.^{4a,4b} Therefore, STn is considered a specific tumor-associated antigen for vaccination. The conjugate of STn antigen with the keyhole limpet hemocyanin (KLH) has been developed as a therapeutic vaccine (Theratope®) for treatment of metastatic breast cancer.⁶ It has been shown that STn with modified sialic acid forms such as those with *N*-iso-butanoyl, *N*-phenylacetyl,⁷ or fluorine⁸ substituent can significantly increase anti-STn IgG titers and induce strong T-cell-mediated immune response in patients.

STn antigens have been synthesized via various chemical approaches.⁹ These methods require multi-step protection and deprotection processes. In addition, the chemical sialylation step often leads to low yields due to difficulties in controlling the stereospecificity of α -sialyl linkage. On the other hand, chemoenzymatic method using sialyltransferase-catalyzed reaction can provide high regio- and stereo- selectivity. Nevertheless, despite of its good expression level in *E. coli* (36 mg/L culture) and high efficiency in sialylation of β -linked galactosides, a previously well characterized recombinant *Photobacterium damsela* α 2–6-sialyltransferase (Pd2,6ST)¹⁰ is much less efficient in using α -*N*-acetylgalactosamine (GalNAc)-linked glycosides as acceptors in synthesizing STn antigen and derivatives.^{10a,10c,11} To improve the yields for the synthesis of STn antigens using chemoenzymatic approaches, we aimed to identify an α 2–6-

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sialyltransferase with good solubility, high expression level in *E. coli*, and has good activity towards α -GalNAc glycosides as acceptors. These criteria are especially important for large scale enzymatic synthesis of STn or preparative synthesis of a library of STn derivatives with diverse sialic acid forms and various aglycons.

Upon analyzing several recently reported bacterial α 2–6-sialyltransferases,¹² we identified the α 2–6-sialyltransferase from *Photobacterium* sp. JH-ISH-224 (Psp2,6ST)^{12a,12b} as our candidate. Psp2,6ST shares 54% amino acid sequence identity with Pd2,6ST and has better expression in *E. coli* compared to the α 2–6-sialyltransferase from *Photobacterium leiognathi* JT-SHIZ-145.^{12c} Also, it does not have the sialidase activity reported for the α 2–6-sialyltransferase from *P. leiognathi* JT-SHIZ-119.^{12d} Using a synthetic gene of Psp2,6ST with codons optimized for *E. coli* expression system as the template for polymerase chain reaction (PCR), a truncated C-His₆-tagged fusion protein with deletions at both N-terminus (deleting 2–14 amino acid residues) and C-terminus (deleting 502–514 amino acid residues) was cloned into pET22b(+) vector. Expression in *E. coli* BL21 (DE3) cells at 20 °C for 20 h with vigorous shaking (250 rpm) after induction with 0.3 mM of isopropyl-1-thio- β -D-galactoside (IPTG) followed by Ni²⁺-NTA column purification routinely provided 25 mg of pure, soluble and active Psp2,6ST(15–501)-His₆ per liter of *E. coli* culture.

The α 2–6-sialyltransferase activity of Psp2,6ST(15–501)-His₆ was investigated under different pH conditions using three fluorescent-labeled acceptors including 4-umbelliferyl β -lactoside (Lac β MU),¹³ GalNAc α 2AA (**11**), and GalNAc α Ser (**12**). As shown in Figure S3 in the Supporting Information, the optimal pH ranges for Psp2,6ST(15–501)-His₆ were pH 7.5–9.0 and pH 7.5–8.0, respectively, when Lac β MU and GalNAc α Ser were used as acceptor substrates. The activity declined quickly when pH was below 6.0 or above 9.0. However, when GalNAc α 2AA was used as the acceptor, the optimal pH range was pH 5.0–6.0. The enzyme activity decreased quickly below pH 5.0 but only decreased moderately with the increase in the pH range of 6.0–10.0. For comparison, His₆-Pd2,6ST(16–497) was active in a wide pH range of 6.0–9.0 when GalNAc α 2AA or GalNAc α Ser was used as an acceptor. This was similar to the pH profile of the enzyme when Lac β MU was used as an acceptor.^{10a} Figure S3 also clearly shows that when GalNAc α 2AA or GalNAc α Ser was used as an acceptor, Psp2,6ST(15–501)-His₆ was a more efficient catalyst than His₆-Pd2,6ST(16–497).

Kinetic studies (Table 1) confirmed that in general, Psp2,6ST(15–501)-His₆ is a more reactive α 2–6-sialyltransferase than His₆-Pd2,6ST(16–497). The catalytic efficiency (k_{cat}/K_m) of Psp2,6ST(15–501)-His₆ was 1.6-, 36.7-, 1.6-, and 3.4-fold higher than that of His₆-Pd2,6ST(16–497) when Lac β MU,^{10b} GalNAc α 2AA (**11**), GalNAc α Ser (**12**), and GalNAc α Thr (**13**) were used as the acceptor substrates respectively. The differences in the catalytic efficiencies were mainly caused by the lower K_m values of different acceptors for Psp2,6ST(15–501)-His₆ compared to His₆-Pd2,6ST(16–497). When GalNAc α 2AA (**11**), GalNAc α Ser (**12**), and GalNAc α Thr (**13**) were used as acceptors, higher k_{cat} values of Psp2,6ST(15–501)-His₆ also contributed to the better catalytic efficiencies especially for the case of GalNAc α 2AA (**11**) for which a 6-fold better k_{cat} value was observed. The kinetic studies thus demonstrated that Psp2,6ST(15–501)-His₆ is a promising catalyst for synthesizing sialyl Tn-antigens.

Using Psp2,6ST(15–501)-His₆ with a *Pasteurella multocida* sialic acid aldolase¹⁴ and an *N. meningitidis* CMP-sialic acid synthetase (NmCSS)¹⁵ in an efficient one-pot three-enzyme sialylation system established in our lab (Scheme 1),¹⁶ sialyl Tn-antigens and derivatives containing different sialic acid forms and various aglycons were synthesized effectively in preparative scales.

To synthesize sialyl Tn analogs containing different sialic acid forms, the reactions were carried out at 37 °C in a Tris-HCl buffer (100 mM) containing Mg^{2+} , pyruvate, CTP, a sialic acid precursor, 3-azidopropyl *N*-acetamido α -galactoside **10** (GalNAc α ProN₃) as a sialyltransferase acceptor, and three enzymes. *N*-Acetylmannosamine **1** (ManNAc), *N*-glycolylmannosamine **2** (ManNGc), mannose **3**, and their chemically synthesized derivatives **4–9** were used as sialic acid precursors. As shown in Table 2, Psp2,6ST(15–501)-His₆ exhibited promiscuous donor substrate specificity and was able to catalyze the transfer of different sialic acids formed from **1–9** to GalNAc α ProN₃ **10** to produce STn analogs **14–22** containing Neu5Ac, Neu5Gc, and other sialic acid forms. The sialosides containing natural occurring sialic acid forms, Neu5Ac α 2–6GalNAc α ProN₃ **14**, Neu5Gc α 2–6GalNAc α ProN₃ **15**, Kdn α 2–6GalNAc α ProN₃ **16**, Neu5GcMe α 2–6GalNAc α ProN₃ **17**, and Neu5GcAc α 2–6GalNAc α ProN₃ **18** were obtained in 60% to 86% yields from ManNAc **1**, ManNGc **2**, mannose **3**, ManNGcMe **4**, and ManNGcAc **5** as six-carbon sialic acid precursors, respectively. Non-natural STn-antigen analogs Neu5AcN₃ α 2–6GalNAc α ProN₃ **19**, Neu5AcF α 2–6GalNAc α ProN₃ **20**, Neu5GcBn α 2–6GalNAc α ProN₃ **21**, and Neu5AcCbz α 2–6GalNAc α ProN₃ **22**, which contain different function groups at the C-5 position of the terminal sialic acid residue, were also obtained in good yields (67–80%) from C2-modified ManNAc derivatives **6–9**. The excellent yield of compound **20** (80%) indicated that the fluorine modification on the *N*-acetyl group of Neu5Ac did not affect the sialyltransferase activity of Psp2,6ST. Fluorine-modification on STn antigen has been shown to significantly increase anti-STn IgG titers and improve the ratios of anti-STn IgG/IgM.⁸ Therefore, fluorinated compounds which provide useful candidates for vaccine development can be obtained efficiently by chemoenzymatic synthesis without compromising the synthetic yield.

STn antigens and analogs containing the most common sialic acid form Neu5Ac and various aglycons were also synthesized. As shown in Table 1, preparative-scale sialylation of GalNAc α 2AA **11** by Neu5Ac formed from ManNAc and pyruvate successfully produced the fluorescent-labeled sialylated product Neu5Ac α 2–6GalNAc α 2AA **23** in 64% yield. The one-pot three-enzyme sialylation of biologically important Tn antigens GalNAc α Ser **12** and GalNAc α Thr **13** was accomplished with 72% and 51% yields, respectively, to produce desired Neu5Ac α 2–6GalNAc α Ser **24** and Neu5Ac α 2–6GalNAc α Thr **25**. In comparison, a previous synthesis of a compound similar to **25** from GalNAc α Thr using the trans-sialidase activity of *V. cholerae* and *C. perfringens* sialidases resulted in low yields (16% and 10% respectively).¹⁷ Compounds **24** and **25** can be used as building blocks in the synthesis of STn glycopeptides. Alternatively, Psp2,6ST(15–501)-His₆ may also be used to improve the synthetic yields of STn peptides via α 2–6-sialylation of Tn peptides previously catalyzed by His₆-Pd2,6ST(16–497)^{10c}.

In summary, we improved the expression of Psp2,6ST in *E. coli* by using a codon-optimized synthetic gene as a template for PCR and introducing an additional C-terminal 13-amino-acid-truncation. The resulting Psp2,6ST(15–501)-His₆ was shown to be a more reactive α 2–6-sialyltransferase than His₆-Pd2,6ST(16–497), especially in the synthesis of STn antigens and their derivatives. Psp2,6ST(15–501)-His₆ exhibited promiscuous donor and acceptor substrate specificities and was used successfully in an efficient one-pot three-enzyme sialylation system for synthesizing STn antigens and derivatives containing diverse natural and non-natural sialic acid forms with various aglycons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

Scheme for one-pot three-enzyme chemcoenzymatic synthesis of sialyl Tn antigens containing different sialic acid forms. Enzymes: Pm aldolase, *P. multocida* sialic acid aldolase; NmCSS, *N. meningitidis* CMP-sialic acid synthetase; Psp2,6ST, *Photobacterium* sp. JH-ISH-224 α 2-6-sialyltransferase.

Table 1

Apparent kinetic parameters of Psp2,6ST(15–501)-His₆ and His₆-Pd2,6ST(16–497).

Enzymes	Psp2,6ST(15–501)-His ₆			His ₆ -Pd2,6ST(16–497)		
	<i>K_m</i> (mM)	<i>k_{cat}</i> (min ^{−1})	<i>k_{cat}/K_m</i> (mM ^{−1} min ^{−1})	<i>K_m</i> (mM)	<i>k_{cat}</i> (min ^{−1})	<i>k_{cat}/K_m</i> (mM ^{−1} min ^{−1})
Substrates						
CMP-Neu5Ac	0.62±0.04	(1.4±0.1)×10 ²	2.2×10 ²	0.90±0.20	(1.6±0.1)×10 ²	1.7×10 ²
LacβMU	0.36±0.07	(1.0±0.1)×10 ²	2.9×10 ²	0.80±0.10	0.14±0.01	1.8×10 ²
CMP-Neu5Ac	3.9±0.4	4.8±0.2	1.2	11±1	0.38±0.01	0.034
GalNAcα2AA	9.5±2.7	31±5	3.3	51±5	4.7±0.2	0.09
GalNAcαOSer	1.4±0.1	34±3	24	1.6±0.2	24±1	15
GalNAcαOThr	5.9±1.6	16±1	2.7	10±1	8.3±0.2	0.8

^a Data were cited from a paper reported previously. ^{10b}

Table 2

One-pot three-enzyme synthesis of STn antigens and derivatives.

Donors	Acceptors	Products	Yields (%)
1	10	14	76
2	10	15	75
3	10	16	60
4	10	17	86
5	10	18	68
6	10	19	75
7	10	20	80
8	10	21	67
9	10	22	72
1	11	23	64
1	12	24	72
1	13	25	51